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This award is a Predoctoral Fellowship to support the doctoral training of Donna Egender. The goal of this research is to investigate the effects of the small stress protein, HSP27, on growth and motility characteristics of normal and tumor-derived human mammary cell lines. Our study is based on the hypothesis that HSP27 is a component of a signal transduction pathway that regulates actin microfilament dynamics. We hypothesize that cells overexpressing HSP27 will show increased motility and altered chemotactic properties, in addition to increased resistance to heat killing and certain drugs. Research completed this year includes selection of 19 clonal MDA231 breast tumor cell lines that overexpress human hsp27, assay of hsp27 expression levels, and determination of the effects of hsp27 overexpression on proliferation rates of the clonal lines and their response to heat stress and drug treatments. Experiments evaluating the effects of hsp27 overexpression on motility of breast tumor cells have been initiated.

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Aileen Hickey 12/9/98
PI - Signature Date

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INTRODUCTION

This award is a Predoctoral Fellowship to support the doctoral training of Donna Egender.

The goal of this research is to investigate the effects of the small stress protein, HSP27, on growth and motility characteristics of normal and tumor derived human mammary cell lines. Preliminary clinical studies indicate that elevated levels of HSP27 in breast tumor cells correlates with aggressive metastasis and poor prognosis (1,2). We have shown that HSP27 overexpression confers resistance to killing by hyperthermia and by certain anti-tumor drugs (3,4). Phosphorylation of HSP27 increases rapidly in cells treated with heat, cytokines or mitogens (5,6,7,8). In rodent cells overexpressing human HSP27, the actin cytoskeleton is resistant to damage caused by hyperthermia or cytochalasin D treatment (9,10). High levels of HSP27 also correlate with increased accumulation of cortical actin, suggesting a possible effect on cellular motility. In contrast, cells expressing a non-phosphorylatable form of HSP27 show inhibition of processes depending on cortical microfilament dynamics (10).

Our study is based on the hypothesis that HSP27 is a component of a signal transduction pathway that regulates actin microfilament dynamics, and may affect cell migration and the metastatic potential of tumors. We propose that cells overexpressing HSP27 will show increased motility and altered chemotactic properties, in addition to increased resistance to heat killing and to certain drugs. Overexpressing cells may respond more vigorously to chemotactic agents, or may respond to different signaling molecules than the parent cell type. We predict that the cells expressing antisense HSP27 sequences, or those expressing the unphosphorylatable mutant will show responses antagonistic to those shown by cells overexpressing normal HSP27.

We proposed to 1) prepare human mammary cell lines expressing either increased levels of HSP27, unphosphorylatable HSP27, or antisense sequences that reduce endogenous HSP27 expression; 2) assay the rate of cell proliferation in these cell lines, compared to controls; 3) assay motility and response to cytokines of these cells using the Boyden chamber technique; and 4) study the resistance of the cell lines to hyperthermia, arsenate, cytochalasin D, and antitumor drugs.

PROGRESS REPORT

I. ACADEMIC:

This award is a Predoctoral Fellowship to support the doctoral training of Donna Egender. Donna has completed all required academic course credits for her degree. Her research has progressed during the past year, and should be completed by Summer 1999. This is therefore not a final report. The final report will be submitted following her defense of Dissertation. During the past year Donna attended the "Breast Cancer Research Program: An Era Of Hope" Meeting in Washington DC, Oct 1997, and presented a poster entitled "Regulation of the Small Heat Shock Protein Gene in Mammary Tumor Cell Lines" on work reported in a previous annual report. The research progress report that follows was written by Donna.

II. RESEARCH:

The main goal of this project was to investigate the effect of overexpression of *Hsp27* on the phenotype of breast cancer cell lines. The specific aims of this project were to 1) prepare human mammary cell lines expressing either increased levels of Hsp27, unphosphorylatable Hsp27, or antisense sequences that reduce endogenous Hsp27 expression; 2) assay the rate of cell proliferation in these cell lines, compared to controls; 3) assay motility and response to cytokines of these cells using the Boyden chamber technique; and 4) study the resistance of the cell lines to hyperthermia, arsenate, cytochalasin D, and antitumor drugs. We also investigated estrogen regulation of the *hsp27* promoter, while cell lines were being established. We found that the estrogen receptor and the suspected estrogen response elements (ERE) are not directly involved in induction of the *hsp27* promoter. These results were reported in a previous progress report (1996).

In previous progress reports we indicated that we developed a limited number of clonal cell lines overexpressing Hsp27 in MDA-231 cells, with much difficulty. We now have established more cell lines and have initiated studies of the effect of Hsp27 on their thermoresistance, proliferation rate, drug resistance, and motility.

Developing clonal cell lines overexpressing human Hsp27

We established additional clonal lines by using a lower concentration of puromycin for selection. Two plasmids were used to transfect into MDA-231 breast tumor cells, one containing the *hsp27* gene under the control of the SV40 promoter (SV2711), and a second containing the *hsp27* cDNA under the control of the β-actin promoter (HβSL1). Each of these was transfected into MDA-231 cells using Lipofectin, and transfectants were selected and maintained using 2.5 μg/ml puromycin. Surviving colonies were trypsinized, pooled, and then replated for selection. Clones were isolated and expanded. Expression of Hsp27 in each clonal line was measured by Western blot of cell lysates. Several clonal lines were found which express Hsp27 at high levels compared to controls. We found nine clonal lines expressing Hsp27 from HβSL1, and eight lines expressing from SV2711. The SV2711 clonal lines expressed a 10-fold increase in Hsp27 over controls, whereas the HβSL1 clonal lines express only 2-3-fold more Hsp27 than the control (Fig. 1). We now had a significant number

of cell lines expressing Hsp27 at differing levels. This will allow us not only to look at the effects of overexpression, but also to investigate the effects of varying amounts of the protein.

Thermoresistance of Hsp27 overexpressing cell lines

We assayed the effect of Hsp27 overexpression on thermoresistance. We assayed all the clonal cell lines showing increased expression of Hsp27, a KS vector transfected clonal line, the untransfected parental control, MDA-231, and MCF7 breast tumor cells, which naturally overexpress Hsp27. Cells ($2x10^4$) were plated into 24-well plates, and placed into a 44°C water bath for 1, 2, 3, or 4 hours, and then allowed to recover at 37°C for two weeks. Surviving cells were stained and visually evaluated for their survival. Figure 2 is a photograph of representative results from this assay, showing control cells and the clonal cell lines, KS-1, SV27-18, and HβSL1-22. The SV2711 clones, which express the highest levels of Hsp27, showed the most thermoresistance. The HβSL1 clones also showed thermoresistance compared to controls, although they were more sensitive than the SV2711 clones.

To quantitate the thermal resistance seen in the previous experiment, we used the clonal cell line SV27-18 to compare its clonal survival following heat stress to that of a KS control cell line, parental MDA-231 cells, and thermotolerant cells. Cells were made thermotolerant by exposing cultures of each of the cell lines to a 44°C heat shock, followed by 16 hours of recovery at 37°C. Thermotolerant and non-thermotolerant cells were subjected to a 44°C heat treatment for 1, 2, 3, or 4 hours. The heat treated cells were counted and replated at known concentrations. After ten days, the cells were stained and surviving colonies were counted. The clonal cell line SV27-18 showed 500,000-fold resistance to heat compared with controls, and only 10-fold less thermoresistance than fully thermotolerant SV27-18 cells (Fig. 3). These results show that the expression of Hsp27 in MDA-231 cells can confer resistance to heat, which suggests that Hsp27 may help cells to survive the physiologically stressful conditions found in many tumors.

Proliferation rate of Hsp27 overexpressing clonal cell lines

Growth rate was assessed in Hsp27 overexpressing clonal cell lines, parental cell lines, and pooled transformant cell lines. Cells ($1x10^{4)}$ were plated into 12-well plates in the absence of puromycin. Triplicate samples were counted daily for 5 days, using a hemacytometer, and media was replaced daily on remaining cells. The number of cells per well was determined and growth rate was measured by plotting cell number per day. Average doubling times were calculated and plotted. We found no significant differences in growth rates between Hsp27 overexpressing clones and control clonal cell lines. We concluded from this that Hsp27 has no significant effect on the growth rate of MDA-231 cells.

Drug resistance of Hsp27 overexpressing clonal cell lines

The survival of several clonal cell lines that had shown heat resistance was measured after exposure to cisplatin, doxorubicin, H_2O_2, or sodium arsenite. We originally proposed to also assay resistance to cytochalasin-D, however the parental MDA-231 cell line was not susceptible to this drug. Optimal concentrations of the cytotoxic agents were determined using the parental cell lines, MDA-231 and

MCF7 in survival studies (data not shown). Using the range of drug concentrations determined in these assays, we exposed three HβSL1 clones, five SV2711 clones, one KS clone, and the MDA-231 parental cell line to increasing amounts of each agent for 1 hour. Cells were trypsinized, counted, and replated at known cell numbers in drug-free media. Cells were allowed to recover for 10 days, and surviving colonies were stained with Coomassie blue and counted. The concentration of drug resulting in 50% survival (IC50) was calculated and results were plotted on a bar graph (Fig. 5). No significant correlation between Hsp27 expression and survival among these cell lines was found for any of the agents tested. We concluded from these experiments that an increased level of Hsp27 in these cell lines does not contribute to resistance to any of the drugs tested.

Effect of Hsp27 overexpression on motility of transfectant cell lines

We have begun preliminary studies to assay the effect of Hsp27 on the motility of tumor cells. A modified Boyden chamber assay (Costar) was used to measure the motility of Hsp27 overexpressing clonal cell lines toward chemoattractants. Cells ($5x10^5$) were plated into a T25, and after 24 hours, cells were fed with serum-free media (DMEM supplemented with ITS: insulin/ transferrin/selenium) for 48 hours. Cells were briefly trypsinized, counted and resuspended in 0.3% BSA/DMEM at a concentration of $8x10^5$ cells/ml. 100 µl of the cell suspension ($8x10^4$ cells) was plated into the top chamber of a collagen I-coated transwell, which was placed into a lower chamber containing DMEM supplemented with 0.03% BSA, with or without 1% FCS as a chemoattractant. Cells were incubated at 37°C for 3 hours, the chambers were disassembled and the cells which migrated to the lower membranes were fixed and visualized by Wright stain. Five visual fields were counted, using a 1 cm^2 grid, and plotted as the average cells/cm^2. The percent stimulation of migration due to attractant was calculated by dividing the number of cells which migrated toward attractant by that which migrated in the absence of attractant (KS clones, Fig. 6; pooled clones, Fig. 7; Hβ clones, Fig. 8; and SV clones, Fig. 9). Our preliminary data suggests a high variability in motility of clonal cell lines. This may be due to several factors, including the fact that some cell lines are physically bigger than others, possibly hindering the passage of some cells through the pores. The only consistency we have seen so far is that the motility without attractant of the clonal cell lines is reduced over the parental cell lines, however we also see this effect with the KS control clonal line. Therefore this decrease in motility may be a general effect of clonal selection.

Future studies

This project will be concluded with several experiments. We will: 1) test motility of clonal cell lines in response to specific chemoattractants such as IGF-I, EGF, and PDGF; 2) evaluate dose response curves and time courses of motility to determine if Hsp27 increases sensitivity to chemoattractant or the kinetics of chemotaxis; 3) measure motility of transiently transfected MDA-231 cells expressing non-phosphorylatable mutant Hsp27 from the following plasmids: SV3XA, SV3XD, and SV3XG (alanine, aspartate, and glycine triple mutants, respectively.); 4) evaluate motility in clonal hamster L929 cell lines containing a Lac-1 inducible hsp27 gene; and 5) determine invasiveness in Matrigel of the Hsp27 overexpressing clonal cell lines.

CONCLUSIONS

This year Donna has proceeded with great focus and persistence, and has been able to make great progress towards completing the studies originally proposed. It is unfortunate that she is not finding significant phenotypic differences between her hsp27 overexpressing clonal lines and the parent cells, other than in heat resistance. She will have a clearer picture when she has completed the migration studies, and assayed cell lines expressing the phosphorylation mutant forms of hsp27. There are a number of possible explanations for the difference in the effects of hsp27 overexpression in MB-MDA231 breast tumor cells and the other cell types reported in the literature. Donna will have to evaluate these possibilities in detail when she has all her data at hand, and will discuss it thoroughly in her dissertation. This will be provided in her final report.

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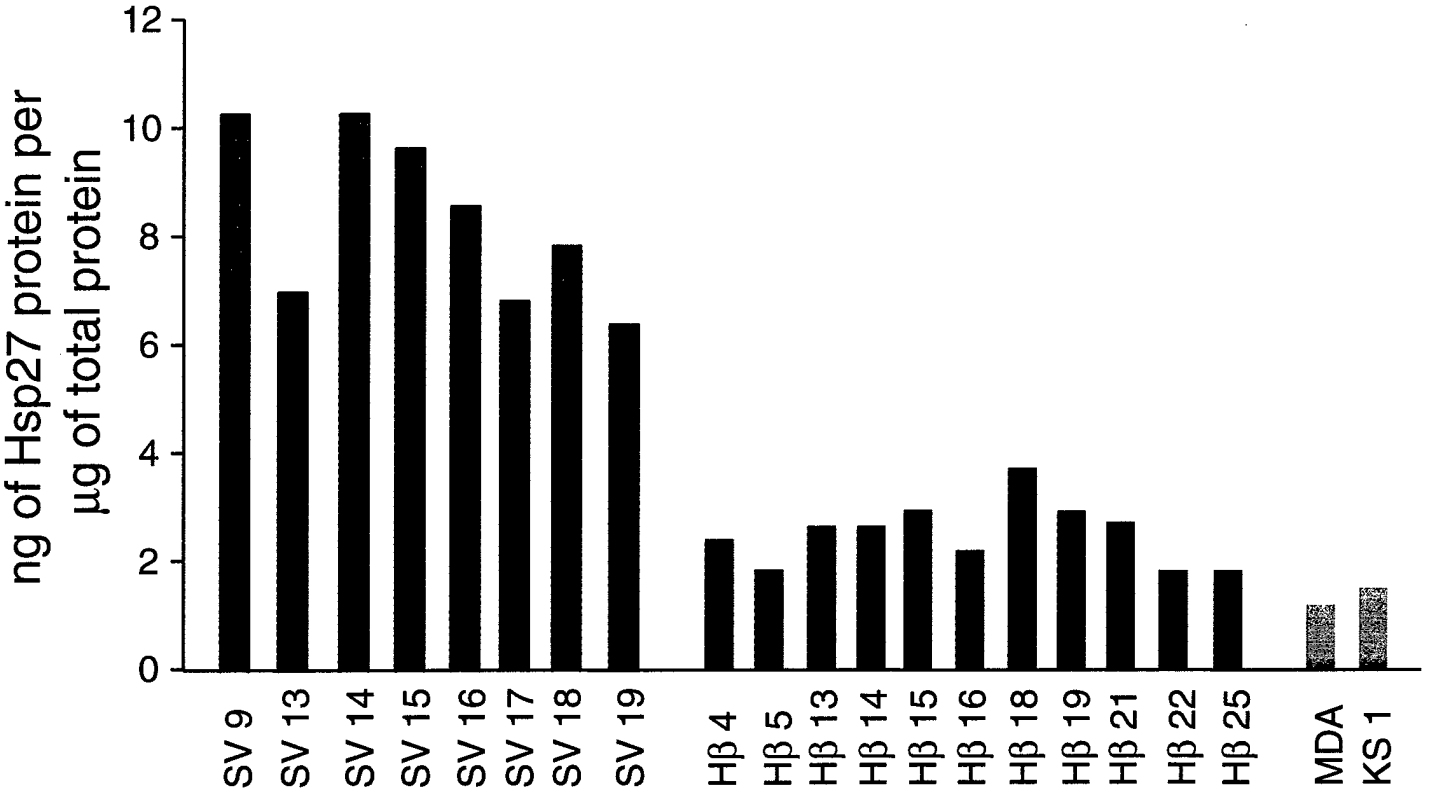

Fig. 1. *Expression of Hsp27 in stably transfected MDA-231 cells.* MDA-231 clonal cell lines were examined for expression of Hsp27 by Western blot, and expression was quantitated by phosphoimager analysis of the membrane. Expression in each clonal cell line is shown as ng of Hsp27 protein per µg of total protein.

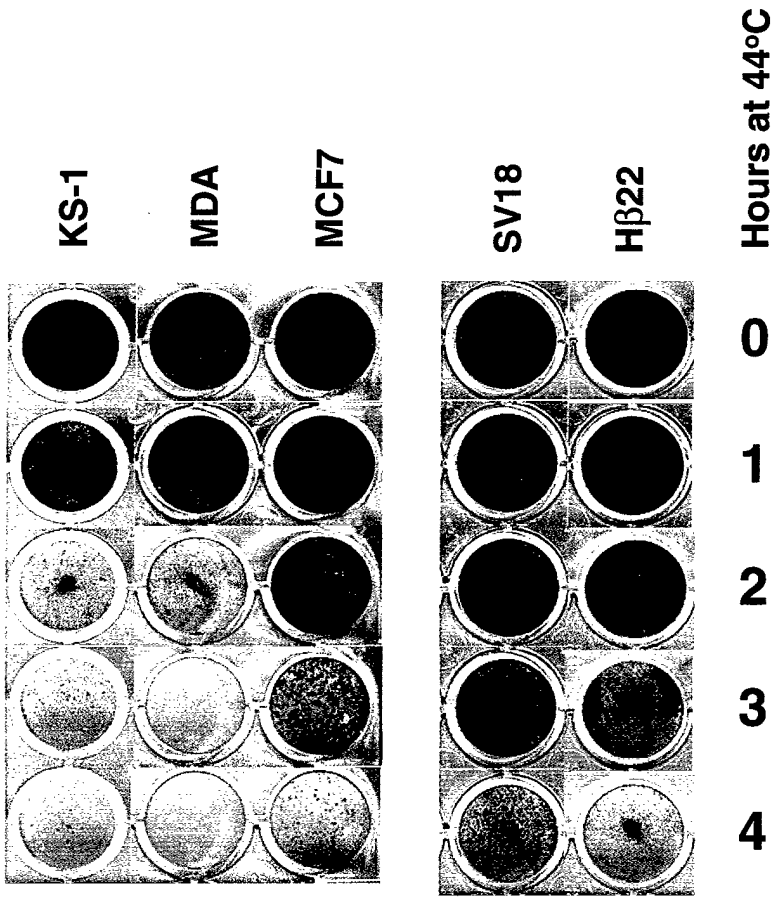

Fig. 2. *Effect of heat treatment on MDA-231, MCF7, and MDA-231 clonal cell lines.* Cells were plated in 24-well plates, and after 24 hours, were heat treated for the indicated times. Cells recovered for 10 days at 37°C, then were stained with Coomasie blue for visualization of surviving cells. The figure shows a representative subset of cell lines; MDA-231, MCF7, KS-1, SV27-18, and HβSL1-22.

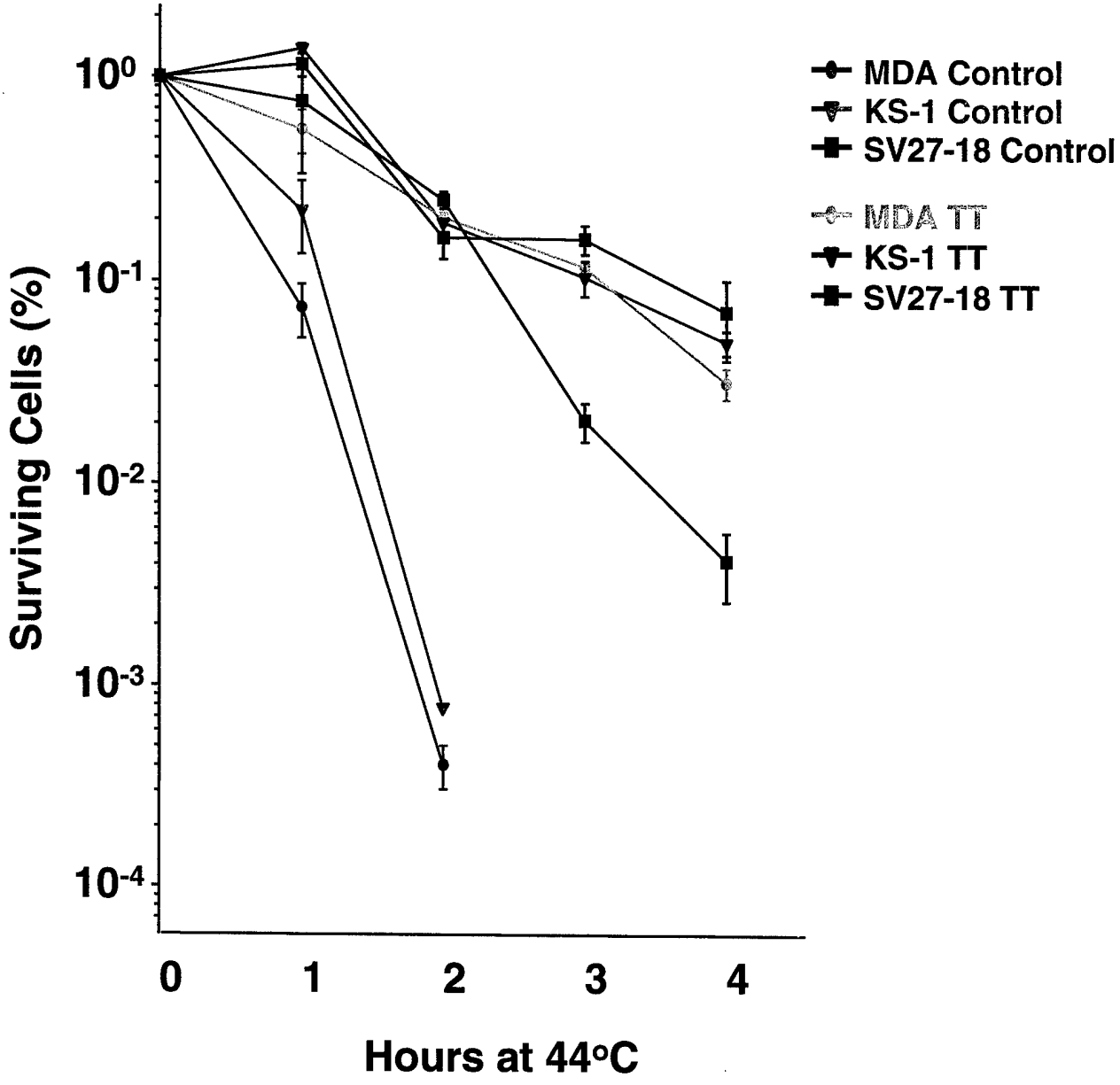

Fig. 3. *Clonal survival following heat stress of untreated and thermotolerant cell lines.* Clonal survival after a heat shock of 44°C for the indicated times is shown for several cell lines: the parental control MDA-231 (—●—), vector-transfected control KS-1 (—▼—), and the Hsp27 overexpressing clonal line SV27-18 (—■—). Cells of each type, which had been made thermotolerant (TT) by a previous mild heat treatment, were also assayed: TT MDA-231 (—◈—), TT KS-1 (—▼—), and TT SV27-18 (—■—). Surviving colonies were stained and counted, and the mean number of surviving colonies ± S.D. was plotted on a log scale.

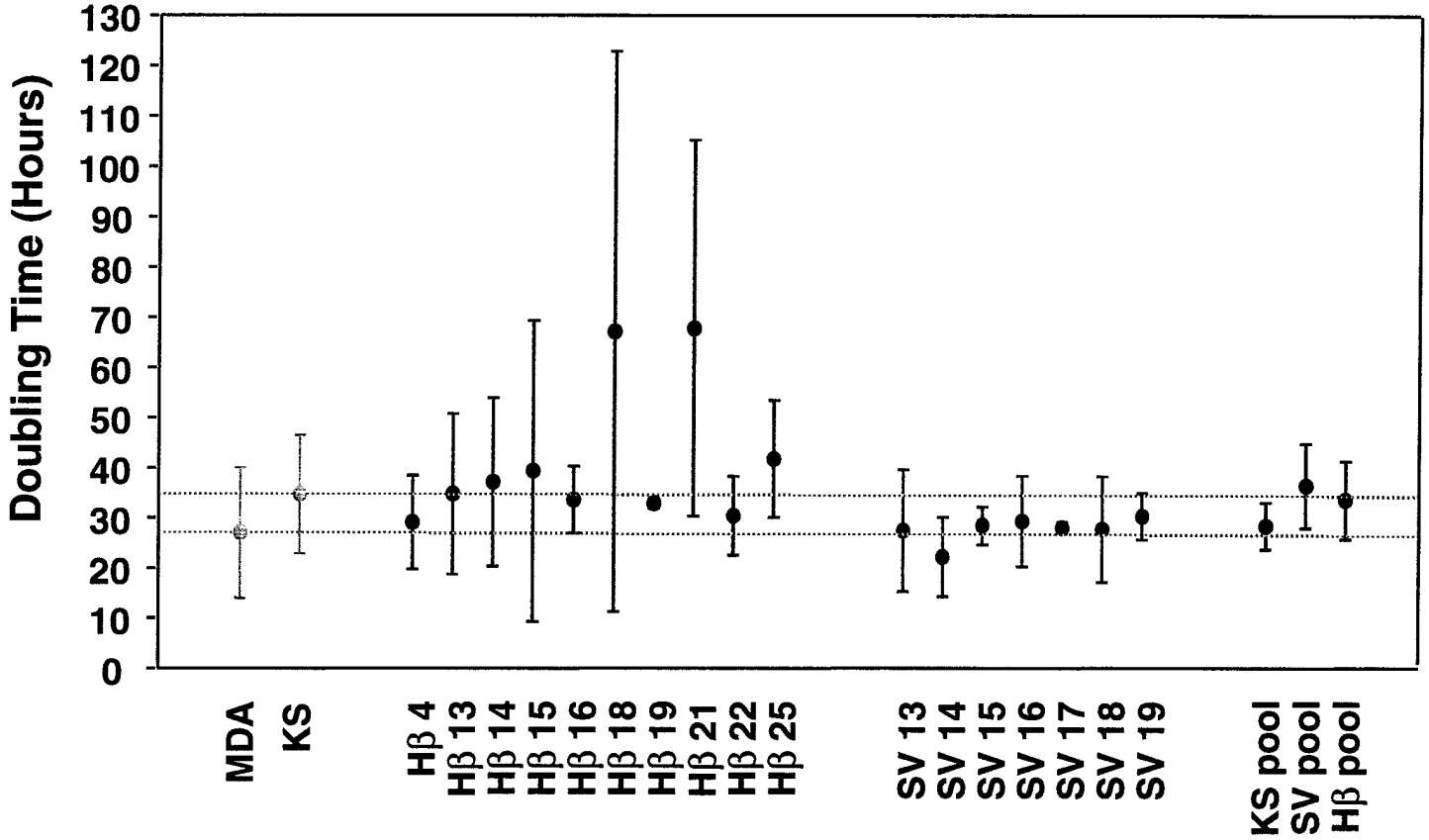

Fig. 4. *Proliferation rate of Hsp27 overexpressing clonal cell lines.* Clonal cell lines were plated into 12-well plates and counted daily. Doubling times (in hours) were calculated and the averages were plotted. The two dotted gray lines represent the average doubling time of the parental control and the KS-1 control.

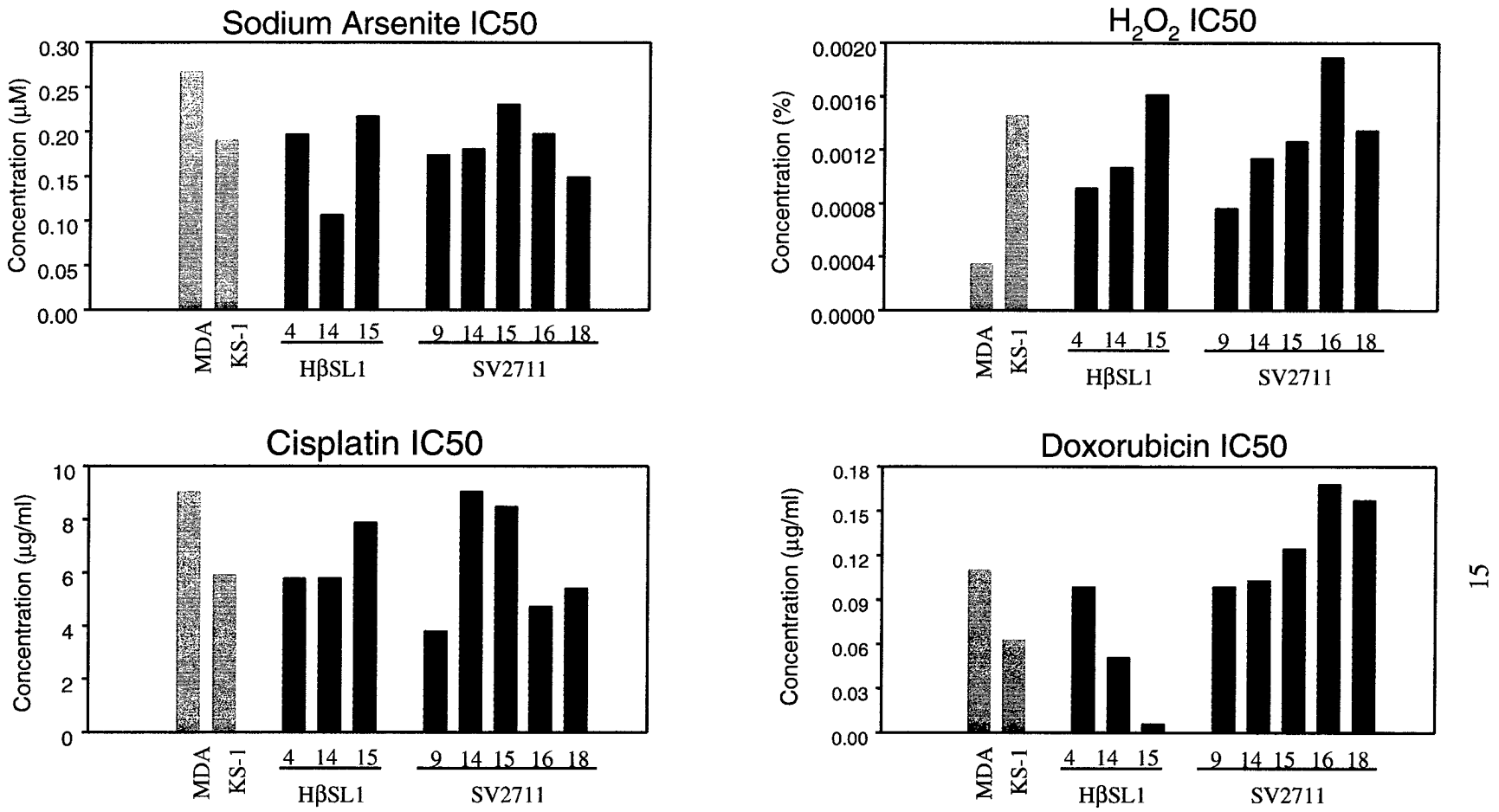

Fig. 5. *Response of clonal cell lines to drug treatments.* The following drugs were used to assess the resistance of cell lines to cytotoxic effects: sodium arsenite, H_2O_2, cisplatin, and doxorubicin. The following clonal cell lines were assayed: Hβ4, Hβ14, Hβ15, SV9, SV14, SV15, SV16, SV18, KS-1, and the parental cell line, MDA-231. Cells ($2x10^5$) were plated into a 6-well plates. After 24 hours, cells were treated for 1 hour with predetermined doses of the each drug, trypsinized, counted, and replated at known cell numbers. After 10 days of recovery in normal media, cells were stained and counted. The IC50 (concentration of drug resulting in 50% survival) was calculated from a plot of concentration of drug vs. % survival. The bar graphs for each drug represent the IC50 for each of the cell lines tested.

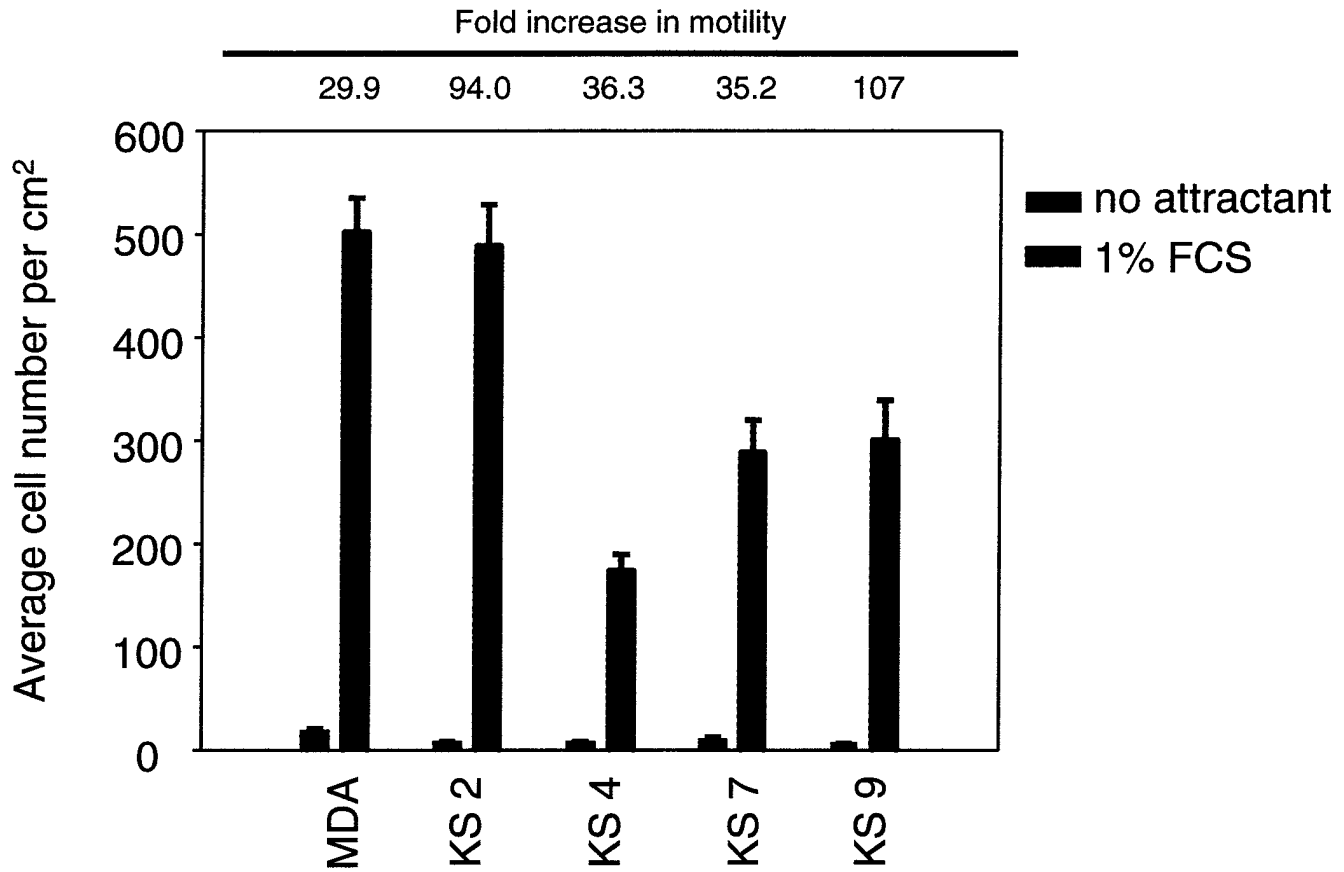

Fig. 6. *Motility of KS clonal cell lines.* Migration of serum-starved clonal cell lines was assayed in response to 1% fetal calf serum. The number of cells indicated is the mean ± S.D. of five visual fields counted per well with either no attractant (▬) or 1% FCS (▬). The fold increase in motility due to attractant is indicated above the graph.

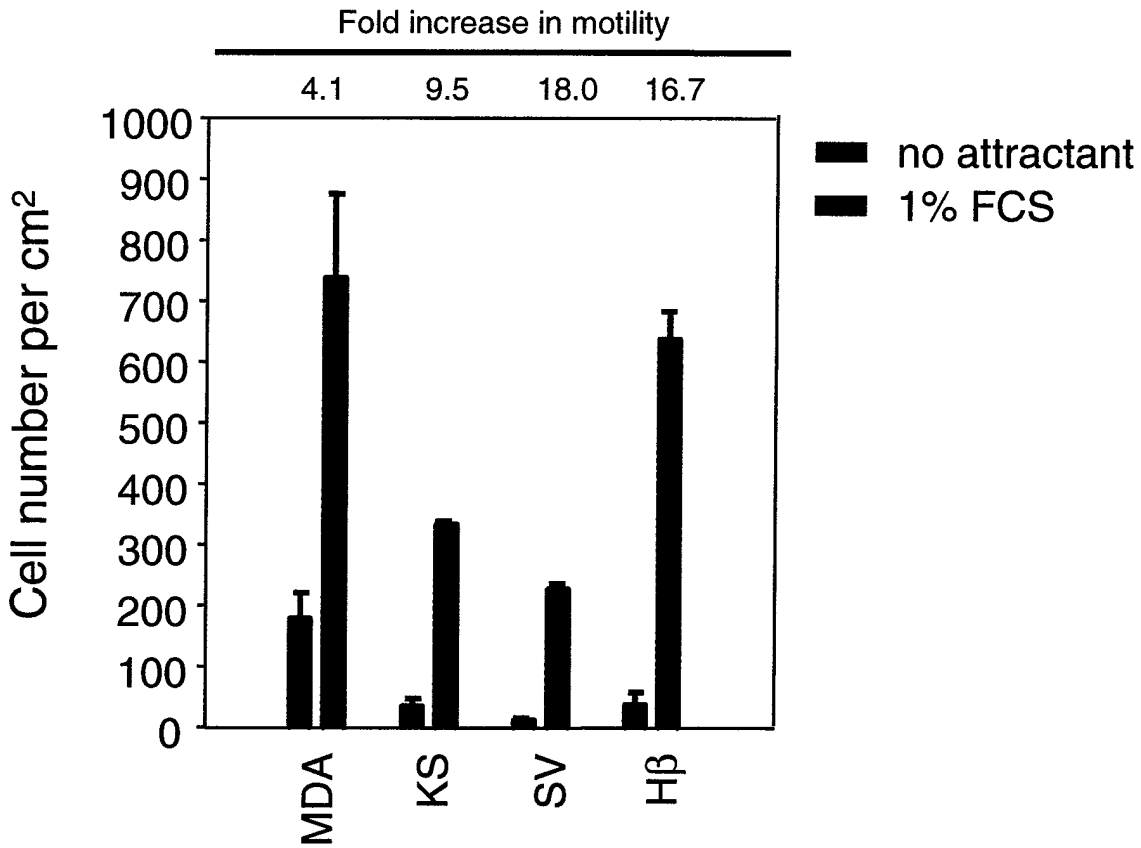

Fig. 7. *Motility of pooled transfected cell lines.* Migration of serum-starved clonal cell lines was assayed in response to 1% fetal calf serum. The number of cells indicated is the mean ± S.D. of five visual fields counted per well with either no attractant (▬) or 1% FCS (▬). The fold increase in motility due to attractant is indicated above the graph.

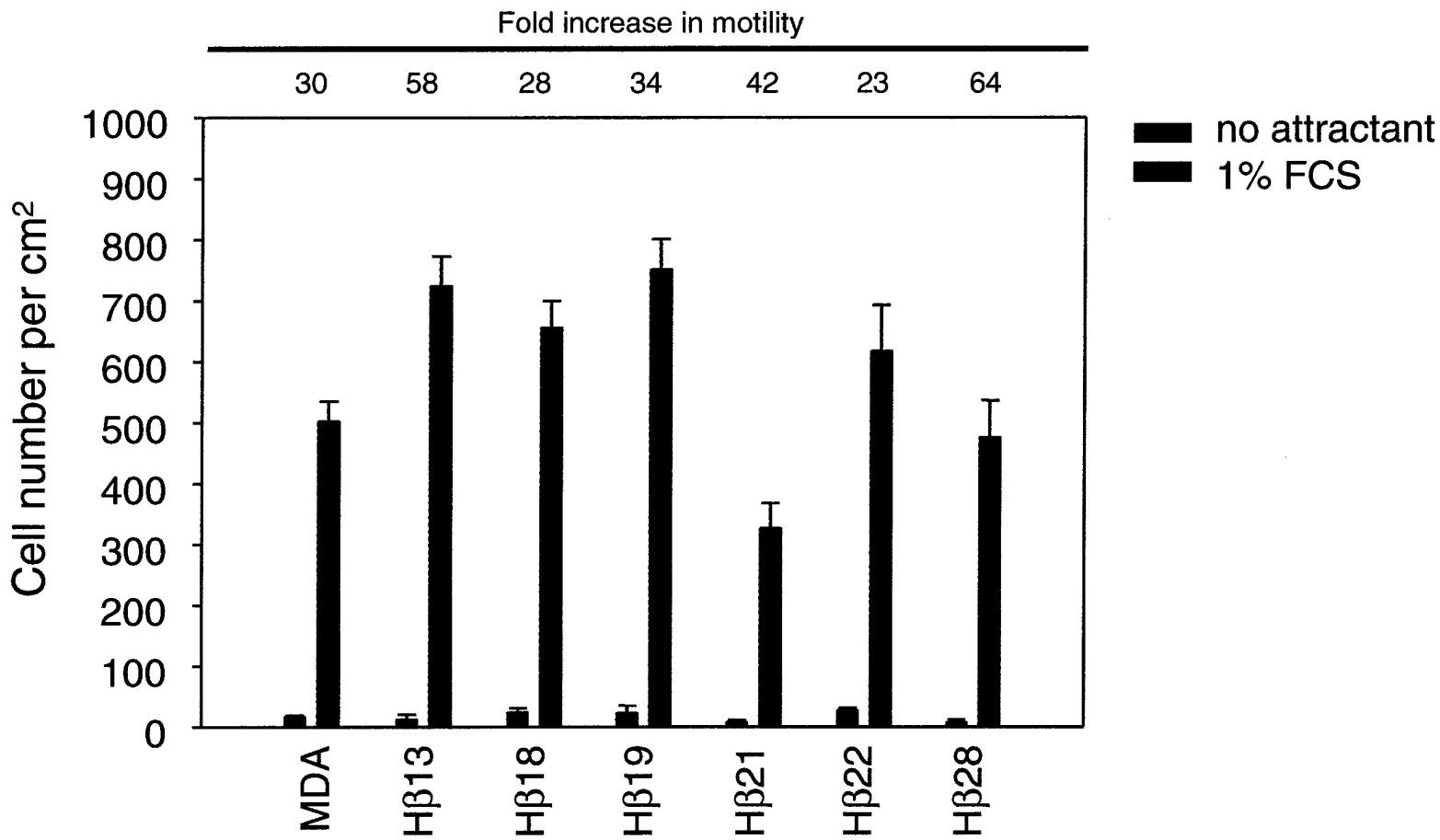

Fig. 8. *Motility of HβSL1 clonal cell lines.* Migration of serum-starved clonal cell lines was assayed in response to 1% fetal calf serum. The number of cells indicated is the mean ± S.D. of five visual fields counted per well with either no attractant (▬) or 1% FCS (▬). The fold increase in motility due to attractant is indicated above the graph.

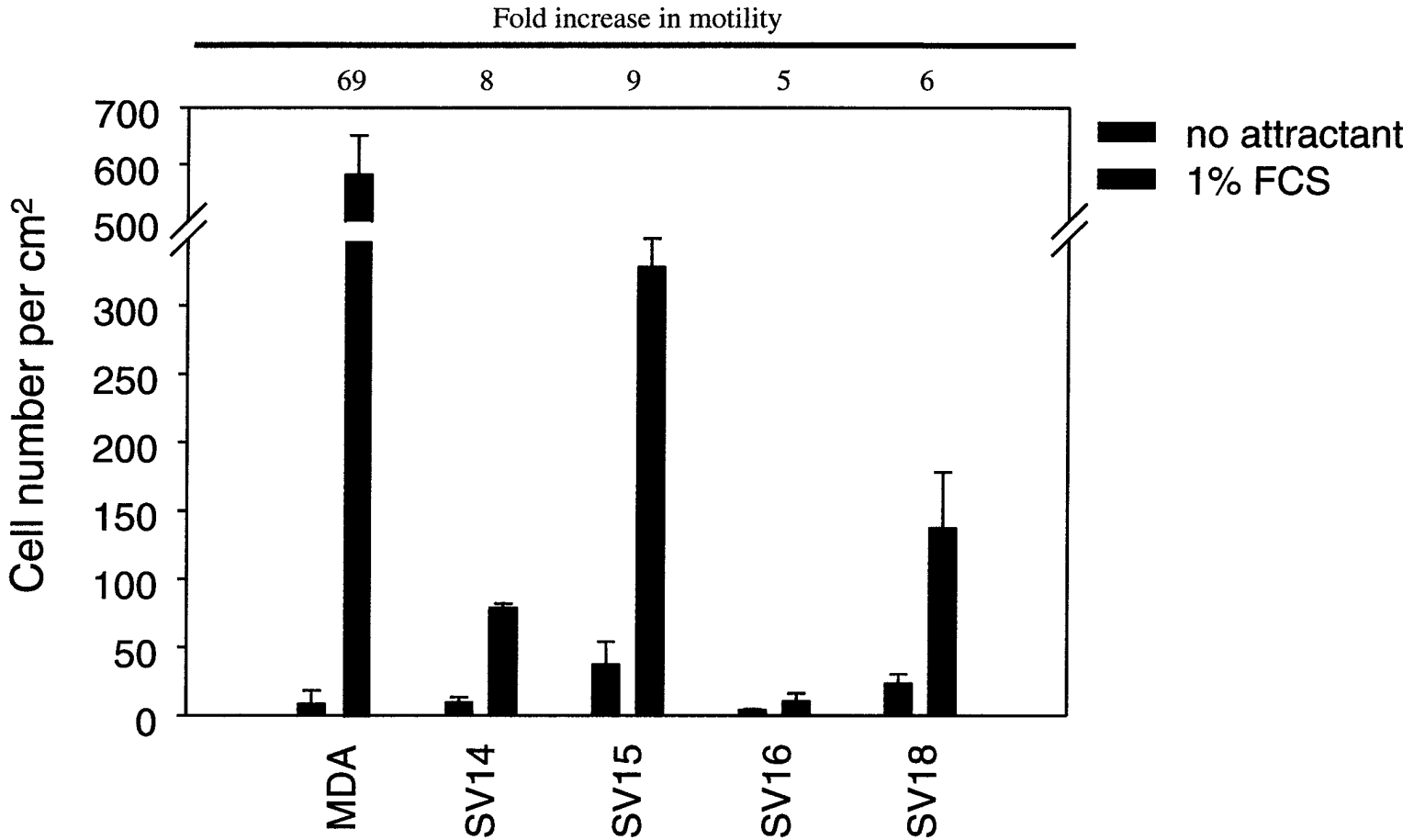

Fig. 9. *Motility of SV2711 clonal cell lines.* Migration of serum-starved clonal cell lines was assayed in response to 1% fetal calf serum. The number of cells indicated is the mean ± S.D. of five visual fields counted per well with either no attractant (▬) or 1% FCS (▬). The fold increase in motility due to attractant is indicated above the graph.

REGULATION OF THE SMALL HEAT SHOCK PROTEIN GENE IN MAMMARY TUMOR CELL LINES

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The small heat shock protein, Hsp27, is a phosphoprotein which increases drug and heat resistance in tumor cells, and plays a role in actin filament dynamics and cytoskeletal structure. Hsp27 is expressed at elevated levels in some breast tumors and may contribute to the progression of the disease. High levels of Hsp27 in breast tumors have been associated with an increase in lymphatic/vascular invasion, drug resistance, nodal metastases, and usually a more aggressive tumor. The effect of increased Hsp27 in other tumor types is controversial, and has been shown to be correlated with both poor and good prognosis. Overexpression of human Hsp27 in transfected rodent fibroblast cell lines has been found to increase cell motility (our unpublished data). The cytoskeleton modulating function of Hsp27 may play a role in an increased metastatic potential of tumors expressing high levels of the protein. In order to clarify the role of Hsp27 in the genesis of the neoplastic phenotype, it is necessary to study both the protein function and regulation of the gene.

Most estrogen receptor (ER) positive breast tumor cell lines contain elevated levels of Hsp27. In certain female reproductive tissues, Hsp27 levels can be induced by estrogen, but not in others. The ER is a transcriptional activator, suggesting that estrogen and ER may play a role in the regulation of the *hsp27* gene. The *hsp27* promoter contains two half estrogen response elements (ERE), such as are found in the estrogen inducible ovalbumin gene. These observations raise the possibility that the *hsp27* gene may be regulated by estrogen via the ER. Therefore we initially examined the estrogen inducibility of the *hsp27* gene.

Keywords: Hsp27, Promoter, Estrogen, Estrogen Receptor, MCF7

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